

indefiniteness as applied to Claims 40 and 41, as stated in the Office Action dated October 10, 2001 (Paper No. 7; page 9, section 10). However, all rejections under 35 U.S.C. § 112, first paragraph, and 35 U.S.C. § 103(a) remain, and all claims (*i.e.*, Claims 1-6, 22-27, 29-30, 40-54 and 73-76) remain rejected.

In the present Amendment and Response, Applicants cancel all claims (*i.e.*, Claims 1-6, 22-27, 29-30, 40-54 and 73-76), and insert new Claims 77-95, which are believed to be allowable. Applicants respectfully request entry of the present Amendment after the Final Office Action, and request reconsideration of the Application in view of the new claims. The new claims do not require additional searches or extensive consideration by the Examiner, and are believed to be in *prima facie* condition for allowance or, at least, present the claimed invention in better form for consideration on appeal.

Support for these new claims is found throughout the Specification. These new claims do not deviate from the subject matter recited in the cancelled claims, and do not constitute new matter.

New independent Claim 77 corresponds to cancelled Claim 1, and recites “IgG heavy chain constant region”, “IgE heavy chain constant region” and “IgG inhibitory receptor”, where these new elements were formerly encompassed by now cancelled dependent claims or were not articulated in the original independent Claim 1. The terms “IgG heavy chain constant region” and “IgE heavy chain constant region” find support in the Specification, for example, at page 16, lines 11-20; page 17, line 16 through page 18, line 3; page 23, lines 15-19 and lines 24-28; and page 24, line 28 through page 25, line 5. The term “IgG inhibitory receptor” finds support at page 2, line 28 through page 3, line 13; page 5, lines 11-12; and page 6, lines 22-26.

The remaining new claims each have counterparts in the cancelled claims, or are within the scope of the cancelled claims. The cancelled claims which correspond to the new claims, or support the new claims, are:

New Claim	Corresponding Cancelled Claim
78	27
79	24
80	25

New Claim	Corresponding Cancelled Claim
81	26
82	5, 6, 43, 44, 47 and 48
83	1, and see Specification at page 2, line 24 through page 3, line 24; page 10, lines 13-27; page 11, lines 13-26; and page 13, lines 13-26
84	3 and 4
85	30
86	<i>e.g.</i> , 30 and 46, and see Specification at page 6, lines 4-5; and page 24, line 28 through page 25, line 5
87	29 and 46
88	<i>e.g.</i> , 42, and see Specification at page 8, lines 19-20; page 22, lines 22-24 and FIG. 3
89	47, where “comprises” has been changed to “consists”
90	<i>e.g.</i> , 42, and see Specification at page 8, lines 25-26; page 23, lines 6-14 and FIG. 6
91	73
92	74
93	53
94	75, and see Specification at page 16, lines 11-15; and page 26, lines 5-10
95	76, and see Specification at page 16, lines 11-15; and page 26, lines 5-10

The previously pending (now cancelled) claims stood rejected for allegedly lacking enablement and written description (35 U.S.C. § 112, first paragraph), for the alleged insertion of new matter (35 U.S.C. § 112, first paragraph), and for alleged obviousness (35 U.S.C. § 103(a)). The Examiner’s rejections are discussed in view of the new claims.

Rejections under 35 U.S.C. 112, First Paragraph, ENABLEMENT

In the Final Office Action, the Examiner maintains his rejection of Claims 1-6, 22-27, 29-30, 40-52, 54 and 73-76 for allegedly lacking enablement (35 U.S.C. § 112, first paragraph) [see

Final Office Action, pages 2-6]. The Examiner alleges that the specification does not provide enablement commensurate in scope with the claims without undue experimentation, and states that the Applicants' arguments were considered and found not persuasive.

Applicants must respectfully disagree with the Examiner's rejection. However, solely for the purpose of furthering Applicants' business interests and advancing the prosecution of the present application, and while reserving the right to prosecute the original or similar claims in the future without loss of equivalents, Applicants have cancelled all previously pending claims, and inserted new Claims 77-95.

New independent Claim 77 now recites a fusion molecule comprising an "IgG heavy chain constant region sequence capable of binding to an IgG inhibitory receptor" and "an IgE heavy chain constant region sequence capable of binding to an IgE receptor." Specific IgG heavy chain constant region and IgE heavy chain constant region sequences with the requisite binding properties are taught in the specification. The specification also teaches that the CH2-CH3 interface of the IgG Fc domain contains the binding sites for the Fc γ RIIb IgG inhibitory receptor (page 21, lines 13-15). The specification further teaches that six amino acid residues (Arg-408, Ser-411, Lys-415, Glu-452, Arg-465, and Met-469) of the human IgE heavy chain CH3 domain are involved in binding to the high affinity IgE receptor, Fc ϵ RI, and that residues, including His, in the C-terminal region of the ϵ -chain make an important contribution toward the maintenance of high-affinity interaction between IgE and Fc ϵ RI (page 21, lines 16-26). This teaching provides valuable guidance for the skilled artisan to design fusion molecules within the scope of the invention that differ from those specifically disclosed. One skilled in the art would understand that regions within the IgG/IgE constant region sequences required for binding to the respective receptors should be retained, or altered with utmost care, while other regions/sites within the native IgG/IgE Fc regions may be deleted or more readily altered, without compromising IgG/IgE binding.

In conclusion, based on the teaching provided in the present specification, and also in view of general knowledge possessed by those skilled in the art, at the priority date of the present application one skilled in the art would have been able to make and use the invention within the full scope of the claims currently pending, without undue experimentation. Accordingly, Applicants respectfully request reconsideration and withdrawal of the enablement rejection under 35 U.S.C. § 112, first paragraph.

Rejections under 35 U.S.C. 112, First Paragraph, WRITTEN DESCRIPTION

In the Final Office Action, the Examiner maintains his rejection of Claims 1-6, 22-27, 29-30, 40-52, 54 and 73-76 for allegedly lacking written description (35 U.S.C. § 112, first paragraph) [see Final Office Action, pages 6-8]. The Examiner alleges that there is insufficient written description in the Specification for the same fusion molecules that were rejected on the basis of lack of enablement, and states that the Applicants' arguments were considered and found allegedly not persuasive.

Applicants must respectfully disagree with the Examiner's rejection. However, without acquiescing to the Examiner's arguments, Applicants have cancelled all previously pending claims, and inserted new Claims 77-95.

For the reasons discussed above, in response to the "lack of enablement" rejection, one skilled in the art at the priority date of the present application would have reasonably concluded that Applicants were in the possession of the invention as currently claimed. Accordingly, Applicants respectfully request reconsideration and withdrawal of the present written description rejection under 35 U.S.C. § 112, first paragraph.

Rejections under 35 U.S.C. 112, First Paragraph, NEW MATTER

In the Final Office Action, the Examiner maintains his rejection of Claims 1-6, 22-27, 29-30, and 40-54 for allegedly containing new subject matter, and alleges that the phrase "other than an antibody variable region" represents a departure from the specification and claims as originally filed (35 U.S.C. § 112, first paragraph) [see Final Office Action, page 8]. The Examiner also states that the Applicants' arguments were considered and found allegedly not persuasive.

Applicants do not agree with the Examiner's assessment. Applicants again note that Claims 53 and 54 appear to be erroneously included in this rejection. Without acquiescing to the Examiner's rejection, Applicants have cancelled all previously pending claims, and inserted new Claims 77-95. The phrase, "other than an antibody variable region" is not recited in the new claims, and thus, the Examiner's rejection is moot. In view of the new claims, applicants respectfully request withdrawal of this rejection.

Claim Rejections Under 35 U.S.C. § 103(a)

In the Final Office Action, the Examiner maintains his rejections of Claims 1-6, 27, 29-30, 40-43, 47-48 and 73-76 for alleged obviousness [35 U.S.C. § 103(a)] over U.S. Patent No. 5,336,603 (issued August 9, 1994) in view of Krauss *et al.* (*Eur. J. Immunol.*, 25:192-199 [1995]) and Basu *et al.* (*J. Biol. Chem.*, 268(18):13118-13127 [1993]), and in view of said same references further in view of WO 88/09344 (published 1988), U.S. Patent No. 5,925,351 (issued July 1999) or Stevenson *et al.* (*Jour. Immunol.*, 158(5):2242-2250 [1997]). The Examiner alleges that this combination of references renders the invention obvious, and further states that the Applicants' arguments were considered and found not persuasive [see Final Office Action, pages 9-18].

The rejection is vigorously traversed.

The Examiner has improperly combined references to arrive at the present invention, since no motivation, either explicit or implied, can be discerned from the references to make such a combination to arrive at the fusion molecules of the present invention. Applicants, again, point out that a proposed combination of references is improper if the combination renders the prior art unsatisfactory for its intended purpose or changes the principle of operation of a reference (see MPEP 2143.01 and 2145), as would be the case if the present combination were made. As the Examiner's combination of U.S. Patent No. 5,336,603, Krauss *et al.* and Basu *et al.* is improper, Applicants argue that supplementation of the combination with any additional prior art teachings is still improper.

Furthermore, even if the cited references could be properly combined, the Examiner failed to make a *prima facie* showing that their combination would make obvious the invention claimed. The claimed invention is directed to fusion molecules which are capable of cross-linking an IgG inhibitory receptor with an IgE receptor (e.g. Fc ϵ RI). Applicants also teach and demonstrate that these fusion molecules unexpectedly find utility in the prevention and treatment of various immune disease, including allergic reactions. While the cited references establish that similar antibody-like structures (immunoadhesins) were known in the art at the time the present invention was made they provide no expectation that the fusion molecules of the present invention would possess valuable properties enabling their use in allergy therapy. The primary references concern CD4-immunoglobulin fusions, where the CD4 molecule has no known involvement in allergy treatment. The secondary references provide no motivation to create

fusion molecules cross-linking an IgG inhibitory receptor with an IgE receptor, or any reasonable expectation that such fusion molecules, if created, would find utility in the treatment of immune diseases, including allergy management. Accordingly, the cited combination, even if properly made, would not make obvious the present invention.

Finally, Applicants also point to secondary considerations that further support the non-obvious nature of the present invention. For example, the structure and function of the fusion molecules of the present invention have been recognized by those skilled in the art as unexpected, and worthy of publication in a prestigious peer-reviewed scientific journal. The fusion molecules of the present invention are described by the present inventors and published in *Nature Medicine* (Zhu *et al.*, “A Novel Human Immunoglobulin Fc γ -Fc ϵ Bifunctional Fusion Protein Inhibits Fc ϵ RI-mediated Degranulation,” *Nature Medicine* 8(5):518-521 [2002]). The “Guide to Authors” section of that journal reads “*Nature Medicine* favors those submissions that represent a conceptual advance or an original approach to understanding the molecular basis of pathogenesis or to developing new therapies, diagnostic procedures and a greater understanding of human disease.” Description of the present invention in such a journal illustrates that those of skill in the art regard the invention as a novel, non-obvious original approach to the treatment of immune diseases.

Without acquiescing to the Examiner’s rejection, Applicants have cancelled all previously pending claims, and inserted new Claims 77-95. Applicants argue that these new claims are allowable, and respectfully request withdrawal of this rejection and request that the claims be passed to allowance.

SUMMARY

For the reasons set forth above, Applicants believe that all new claims (*i.e.*, Claims 77-95) pending in this Application are allowable. Should the Examiner believe that a telephone interview would expedite the prosecution of this Application, Applicants invite the Examiner to call the undersigned attorney at the telephone number indicated below.

Please charge any additional fees, including any fees for extension of time, or credit overpayment to Deposit Account No. 11-1410. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

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ARTICLES

A novel human immunoglobulin Fc γ -Fc ϵ bifunctional fusion protein inhibits Fc ϵ RI-mediated degranulation

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Human mast cells and basophils that express the high-affinity immunoglobulin E (IgE) receptor, Fc ϵ receptor 1 (Fc ϵ RI), have key roles in allergic diseases. Fc ϵ RI cross-linking stimulates the release of allergic mediators¹. Mast cells and basophils co-express Fc γ RIIb, a low affinity receptor containing an immunoreceptor tyrosine-based inhibitory motif and whose co-aggregation with Fc ϵ RI can block Fc ϵ RI-mediated reactivity^{2,4}. Here we designed, expressed and tested the human basophil and mast-cell inhibitory function of a novel chimeric fusion protein, whose structure is γ Hinge-CH γ 2-CH γ 3-15aa linker-CH ϵ 2-CH ϵ 3-CH ϵ 4. This Fc γ -Fc ϵ fusion protein was expressed as the predicted 140-kD dimer that reacted with anti-human ϵ - and γ -chain specific antibodies. Fc γ -Fc ϵ bound to both human Fc ϵ RI and Fc γ RII. It also showed dose- and time-dependent inhibition of antigen-driven IgE-mediated histamine release from fresh human basophils sensitized with IgE directed against NIP (4-hydroxy-3-iodo-5-nitrophenylacetyl). This was associated with altered Syk signaling. The fusion protein also showed increased inhibition of human anti-NP (4-hydroxy-3-nitrophenylacetyl) and anti-dansyl IgE-mediated passive cutaneous anaphylaxis in transgenic mice expressing human Fc ϵ RI α . Our results show that this chimeric protein is able to form complexes with both Fc ϵ RI and Fc γ RII, and inhibit mast-cell and basophil function. This approach, using a Fc γ -Fc ϵ fusion protein to co-aggregate Fc ϵ RI with a receptor containing an immunoreceptor tyrosine-based inhibition motif, has therapeutic potential in IgE- and Fc ϵ RI-mediated diseases.

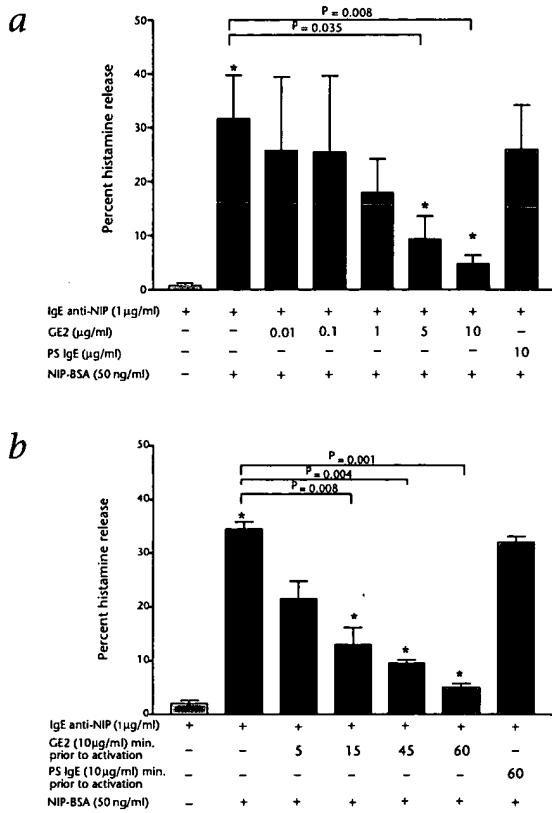
Allergen aggregation of IgE bound to Fc ϵ RI induces release of preformed mediators and synthesis of later acting leukotrienes and chemokines from mast cells and basophils⁵. This process has a major role in many of human diseases such as asthma, allergic rhinitis, chronic urticaria, angioedema and anaphylaxis. Fc ϵ RI aggregation induces release of preformed mediators and synthesis of later-acting leukotrienes, chemokines and cytokines⁵. The Fc ϵ RI is a heterotetramer consisting of a single IgE-binding α -subunit, a β -subunit and two disulfide-linked γ -subunits. The β - and γ -subunit cytoplasmic tails each contain a conserved immunoreceptor tyrosine-based activation motif (ITAM). Cross-linking Fc ϵ RI via IgE bound to multivalent antigen activates tyrosine phosphorylation of ITAMs, thereby initiating downstream signaling⁶. Mast cells and basophils co-express Fc γ RIIb, which contains two extracellular immunoglobulin-like loops and a single conserved immunoreceptor tyrosine-based inhibition motif (ITIM) within its cytoplasmic tail⁷. Fc γ RIIb may co-aggregate with Fc ϵ RI under physiologic conditions. It is

hypothesized that when Fc γ RIIb aggregates, it induces inhibitory signaling via SH2-domain-containing inositol 5-phosphatase (SHIP) and phosphorylation of Fc γ RIIb requires the co-aggregation with Fc ϵ RI (refs. 8,9). Aggregating Fc γ RII to Fc ϵ RI leads to the rapid tyrosine phosphorylation of the Fc γ RIIb ITIM tyrosine by Fc ϵ RI-associated Lyn and inhibition of Fc ϵ RI signaling¹⁰⁻¹³. To take advantage of this Fc γ RIIb-negative signaling in human basophils and mast cells, we constructed a single chimeric human bifunctional protein (GE2) engineered by fusing the human Fc γ 1 and Fc ϵ . Here we show that this chimeric protein is able to inhibit IgE-mediated *in vitro* activation of human basophils and mast cells and *in vivo* activation of human Fc ϵ RI α -bearing mast cells in Fc ϵ RI α transgenic mice. This approach using chimeric Fc γ proteins provides a platform for non-specific as well as future antigen-specific inhibition of IgE-Fc ϵ RI-mediated reactivity in a host of human diseases.

Human genomic DNA encoding the IgG γ 1 constant region extends from the hinge through the CH3 domain. This sequence was initially cloned into a mammalian expression vector with a cytomegalovirus promoter and a murine immunoglobulin κ -chain leader sequence. Genomic DNA of the human ϵ heavy chain CH2 through CH4 domains was placed after the CH γ 3. Between Fc γ 1 and Fc ϵ , we placed a 15 amino-acid linker (Gly₅Ser)₃, which has been used in single-chain Fv fragment expression¹⁴. This flexible peptide linker facilitates chain pairing and minimizes refolding and aggregation problems encountered when the two chains are expressed individually. The fusion protein contained the binding sites for Fc ϵ RI (CH ϵ 2-CH ϵ 3) and for Fc γ RII (CH γ 2-CH γ 3)^{15,16}. The first constant region domains (CH γ 1 or CH ϵ 1) were deleted as this domain associates with BiP (immunoglobulin heavy-chain binding protein) and a fusion protein containing these regions was not expressed¹⁷. SDS-PAGE demonstrated the Fc γ 1-Fc ϵ fusion protein was expressed as the predicted ~140-kD dimer. Western-blot analysis and ELISA testing demonstrated that the GE2 protein was recognized by antibodies specific for human ϵ and γ chains.

Binding of GE2 to both the human Fc ϵ RI and Fc γ RII was demonstrated using CHO3D10, a human Fc ϵ RI α -transfected cell line and HMC-1, which expresses human Fc γ RII but not Fc ϵ RI (ref. 18). GE2 protein bound to both Fc ϵ RI and Fc γ RII in a fashion equivalent to human IgE and IgG, respectively, as assessed by flow cytometry (data not shown). These results demonstrate that expressed GE2 protein is properly folded so as to preserve bifunctional heterotypic FcR binding.

Fresh human basophils expressing both Fc ϵ RI and Fc γ RII can be passively sensitized with chimeric human IgE specific for NIP (4-



hydroxy-3-iodo-5-nitrophenylacetyl) and then histamine release induced by cross-linking the anti-NIP IgE-Fc ϵ RI complex with NIP-BSA (ref. 19). We sensitized basophils with 1 μ g/ml human anti-NIP IgE, plus doses of GE2 ranging from 0.01 to 10 μ g/ml for 1 hour before activation with 50 ng/ml NIP-BSA (Fig. 1a). Myeloma IgE (PS myeloma) was used as a control. We found that 1 μ g GE2 at 1 μ g per ml inhibited almost half of histamine release, whereas at 10 μ g/ml GE2 gave an average of 84% inhibition. 10 μ g of non-specific PS IgE only decreased histamine release by 19%. Adding the GE2 at the same time as the IgE anti-NIP gave optimal inhibition. The longer the delay in GE2 addition following sensitization with IgE anti-NIP, the less the inhibition (Fig. 1b). These results show that the inhibition of antigen-driven histamine release induced by GE2 is dependent on time and dosage. We obtained similar results with cultured human mast cells; a dose of 1 μ g caused 50% inhibition of IgE-mediated degranulation (data not shown). Cross-linking of GE2 by adding an anti-IgG antibody *in vitro* along with GE2 caused enhanced inhibition of IgE-mediated release (data not shown). In performing this experiment, we sought to mimic what may occur *in vivo* should subjects given GE2 make an antibody response against it. The actual fate of antibody bound GE2, should it occur *in vivo*, is not possible to predict.

Tyrosine phosphorylation of Syk is a critical step in human mast cell and basophil mediator release¹⁹. Cross-linking Fc ϵ RI on human basophils with IgE directed to NIP and NIP-BSA induces substantial tyrosine phosphorylation of Syk which was markedly reduced in cells pre-incubated with GE2 (Fig. 2). Thus, GE2 co-aggregation of Fc ϵ RI and Fc γ RII inhibits IgE-mediated Syk phosphorylation, which may contribute to the inhibition of histamine release.

Fig. 1 Dose- and time- dependent inhibition of basophil histamine release using GE2. *a*, Dose-dependence. Results are representative of 3 separate donors, each done in duplicate. *b*, Time-dependence. Results are representative of 2 separate donors, each done in duplicate. For both panels: **sign*ificant differences in histamine release ($P < 0.05$), comparing the two indicated conditions. Total histamine in the donor basophils was 1.2 μ g per 1×10^6 basophils.

Transgenic mice altered to express the human Fc ϵ RI α and with the murine Fc ϵ RI α chain knocked out demonstrate allergic reactivity mediated by passively administered human IgE antibody along with antigen^{20, 21}. The human Fc ϵ RI α chain is coupled with the mouse β and γ chains to form a functional chimeric human/mouse Fc ϵ RI. This model takes advantage of the fact that murine mast cells also express Fc γ RIIb that can interact well with human IgG. This is in contrast to the lack of interaction between the murine Fc ϵ RI and human IgE. We used passive cutaneous anaphylaxis (PCA) to test the GE2 protein's ability to block IgE-driven Fc ϵ RI-mediated mast-cell release. We intradermally primed transgenic mice with 250 ng of chimeric human anti-NP (4-hydroxy-3-nitrophenylacetyl) IgE and simultaneously injected individual sites with saline, GE2 or IgE myeloma protein. Four hours later, mice were given a systemic challenge with 0.5 mg NP-BSA plus 1% Evans Blue intravenously. The intensity of the PCA at each site was assessed by the size of the skin bluing after 30 min. The size and color intensity of the reaction at the sites of GE2 injection were decreased compared with sites injected with an equivalent amount of human IgE myeloma (Fig. 3). Similar results were obtained using genetically engineered human IgE anti-dansyl antibody and dansyl-BSA as an antigen. A total of 30 mice were tested and the GE2 was 2–4 times more potent than purified control human IgE in its ability to block PCA.

Our results demonstrate that a single chimeric molecular human protein consisting of Fc γ 1 plus Fc ϵ directly inhibits *in vitro* histamine release from human basophils and also inhibits humanized Fc ϵ RI-mediated mast-cell degranulation in transgenic mice. GE2 was more potent than control human IgE in all assays. Even if the GE2 protein did not have a 15 amino-acid linker between the two segments of 'normal' human Fc γ 1 and Fc ϵ , it would likely be recognized as foreign and induce an antibody response. With the 15 amino-acid linker, it is highly likely that antibody against GE2 would be expressed and will be primarily directed at this linker region. In fact, such an antibody response to GE2 may well prove advantageous as antibodies against GE2 will lead to increased cross-linking of Fc γ and Fc ϵ receptors and should enhance

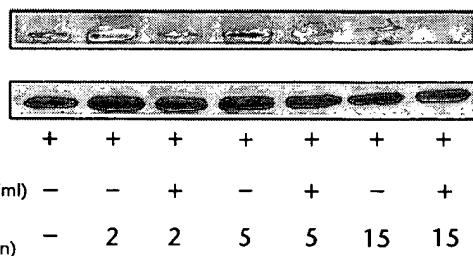


Fig. 2 Co-aggregation of Fc γ RII and Fc ϵ RI by GE2 inhibits Fc ϵ RI-mediated Syk phosphorylation. Immunoprecipitates were analyzed by western blotting with antibody against phosphotyrosine (top row), followed by anti-Syk antibody (bottom row). The top row represents phosphorylated Syk and the bottom row represents total Syk. Each lane shows Syk immunoprecipitated from 5×10^6 basophils. Results represent 2 separate experiments.

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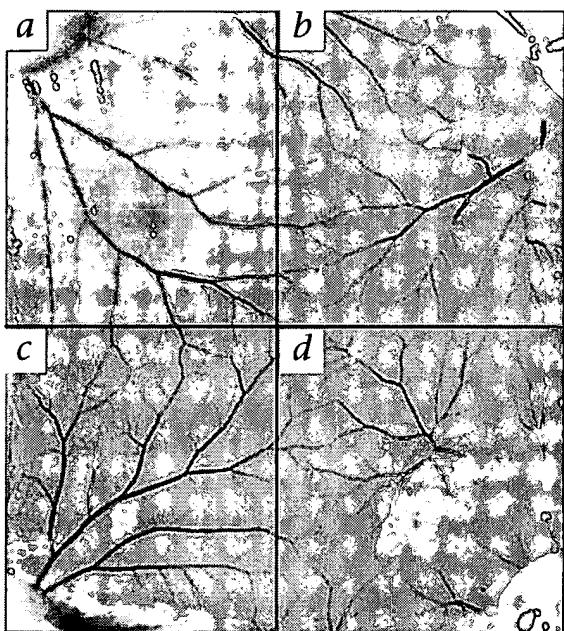


Fig. 3 *In vivo* immunoglobulin Fc γ -Fc ϵ fusion protein inhibits IgE-mediated degranulation in transgenic mice. **a-d**, Mice were injected intradermally with the following: **a**, anti-NP IgE; **b**, saline; **c**, anti-NP IgE and GE2; **d**, anti-NP IgE and IgE myeloma.

inhibition of basophil and mast-cell function as we have observed *in vitro* using an anti-IgG antibody. This truncated Fc γ -Fc ϵ fusion protein, GE2, provides a novel approach for the immunotherapy of diseases mediated via the Fc ϵ R such as allergic asthma, allergic rhinitis, chronic urticaria, angioedema and anaphylaxis. Replacement of the Fc ϵ sequence in our construct with specific allergen genes will result in chimeric bifunctional proteins. We predict that these proteins will have the ability to inhibit mast-cell and basophil reactivity in an antigen-specific manner rather than in the global fashion of GE2. Such novel antigen-specific reagents may be useful in allergen immunotherapy.

Methods

Construction and expression. To construct the human Fc γ -Fc ϵ chimeric gene, the human IgE Fc region (CH2-CH3-CH4) was amplified from pAG vector (provided by S.L. Morrison), containing the whole ϵ genomic DNA. The 5' end primer was 5'-GCTCGAGGGTGGAGCCGGTTCAGCCGAG-GTGGCTCTGGCGGATCGTTCACCCGCCACCGTGAAG-3', containing a flexible linker sequence and a Xhol site. The 3' end primer was 5'-GGCGCCGCTCATTTACCGGGATTACAGACAC-3', containing a NotI site. After amplification, PCR products were cloned into pCR2.1 vector (Invitrogen, Carlsbad, California) and sequenced. Then the Xhol-NotI fragment was inserted into SalI-NotI site of pAN expression vector (from S.L. Morrison), containing the human genomic IgG γ 1 constant region from hinge through the end of CH3. The IgE Fc region was placed downstream of the IgG γ 1 constant region in frame with the CH3 and joined by a (Gly, Ser), flexible linker. The expression vector containing the immunoglobulin Fc γ -Fc ϵ chimeric gene was transfected into SP2/0 cells. The Fc γ -Fc ϵ fusion protein GE2 was expressed in cell-culture supernatants and purified by using an anti-human IgE affinity column.

Western-blot analysis. The purified GE2 protein was run on 7.5% SDS-PAGE and then transferred into Immobilon transfer membranes (Millipore, Bedford, Massachusetts). For protein detection, blots were probed with either goat anti-human IgE (ϵ chain-specific) or goat anti-human IgG (γ chain-specific) conjugated to alkaline phosphatase (KPL,

Gaithersburg, Maryland). Color development was performed with an alkaline phosphatase conjugated substrate kit (BIO-RAD, Hercules, California).

Binding analysis. Human Fc ϵ R binding of the purified GE2 protein was tested by binding to Fc ϵ RI-transfected CHO3D10 cells and HMC-1 cells expressing Fc ϵ RII and then analyzed by flow cytometry (FACSCalibur) using the FITC-conjugated anti-human IgE and anti-human IgG1 reagents (Biosource, Camarillo, California).

Histamine release. Acid-stripped Percoll-enriched human blood basophils were sensitized with chimeric human anti-NIP IgE (10 μ g/ml) at 37 °C in a 5% CO₂ incubator and 1 h later, challenged with 50 ng of NIP-BSA (ref. 19). Histamine release was measured in the supernatants 30 min later. GE2 or control human myeloma IgE was added at various doses and times to test the effects on histamine release.

Measurement of Syk phosphorylation. NIP-sensitized basophils were incubated with or without GE2 (10 μ g/ml), washed and challenged for 2, 5 or 15 min with 50 ng/ml NIP-BSA. Treated or control NIP-IgE sensitized basophils were lysed in 400 μ l of 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Brij, 1 mM sodium orthovanadate and 1 μ g/ml each of antipain, leupeptin, aprotinin and phenylmethanesulfonyl fluoride (PMSF) and incubated for 10 min on ice. Immune complexes were generated by incubating clarified supernatants with anti-Syk pre-adsorbed to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). Proteins were separated by SDS-PAGE, transferred to nitrocellulose and phosphoproteins identified by anti-phosphotyrosine immunoblotting using enhanced chemiluminescence detection reagents (ECL, Amersham, Piscataway, New York). After probing with anti-phosphotyrosine antibody, blots were stripped for 30 min with 100 mM 2-mercaptoethanol, 2% SDS (w/v), and 0.5 M Tris-HCl buffer (pH 6.8) at 50 °C, and then reprobed with anti-Syk antibody (1 μ g/ml).

Passive cutaneous anaphylaxis. Transgenic mice expressing the human Fc ϵ RI α chain and with the murine Fc ϵ RI α chain knocked out²⁰ were primed intradermally with 250 ng of NP-specific recombinant human IgE (ref. 21) in 50 μ l saline. Individual sites were injected with saline, GE2 or IgE myeloma protein simultaneously. 4 h later mice were then given an intravenous challenge with 1.5 mg/ml of NP-BSA plus 1% Evans blue in 300 μ l saline solution. Cutaneous anaphylaxis was assessed visually by the blue dye leakage from blood vessels into the skin.

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Competing interests statement

The authors declare competing financial interests: see the website (<http://medicine.nature.com>) for details.

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